

# Extended-spectrum $\beta$ -lactamase-producing *Escherichia coli* isolated from healthy Thoroughbred racehorses in Japan

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*Extended-spectrum  $\beta$ -lactamase-producing Escherichia coli (ESBLEC) have become a major health concern in both human and veterinary medicine. These bacteria could become a critical problem in equine medicine due to the limited number of antimicrobial drugs available. However, there are no previous reports of ESBLEC isolated from horses in Japan. The objectives of this study were to investigate the occurrence of ESBLEC isolated from feces in healthy Thoroughbred racehorses in Japan. Feces samples were collected from 147 healthy Thoroughbred racehorses by equine veterinarians at the Japan Racing Association (103 from Miho Training Center and 44 from Ritto Training Center) between March 2017 and April 2018. Samples were screened for ESBLECs using MacConkey agar supplemented with 1  $\mu$ g/ml cefotaxime. Detection of ESBL genes was performed by PCR and confirmed by DNA sequencing. Horizontal transmission was demonstrated by conjugation assay. In this study, 24 ESBLECs were isolated from twelve horse feces samples (8.2%). All ESBLECs harbored bla<sub>CTX-M-2</sub>, and both bla<sub>TEM-1</sub> and bla<sub>CTX-M-2</sub> were detected in nine isolates (37.5%). ESBLECs showed resistance to all  $\beta$ -lactam antibiotics (100%) tested, followed by trimethoprim (66.7%), streptomycin (62.5%), tetracycline (25.0%), and oxytetracycline (25.0%). Horizontal transmission was successfully demonstrated by conjugation assay in eight of 13 isolates, and bla<sub>CTX-M-2</sub> was detected by PCR in all transconjugants. This study showed that racehorses in Japan are potential reservoirs of ESBLECs.*

**Key words:** *Escherichia coli*, extended-spectrum  $\beta$ -lactamase, horse

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*Escherichia coli* are commensal intestinal bacteria in most animals and therefore are frequently exposed to antimicrobials administered in the treatment of infections caused by other organisms. This provides the potential for *E. coli* to select resistance determinants from other bacterial species and also to act as a reservoir for resistance genes

[21]. Therefore, commensal bacteria could play roles as both acceptors and donors of antimicrobial resistance (AMR) [1]. Extended-spectrum  $\beta$ -lactamase (ESBL) is one mechanism by which bacteria gain resistance to a wide range of  $\beta$ -lactam antibiotics, including cephalosporins and monobactams, by hydrolyzing their  $\beta$ -lactam ring [20]. After SHV-1 and TEM-1, CTX-M is the most dominant ESBL, and a rapid increase has been reported over the past decade [16, 29, 33]. Furthermore, ESBL genes are easily spreading between different species, which is due to their location on conjugative plasmids [28]. ESBL-producing Enterobacteriaceae are potentially zoonotic bacteria that have received special attention in relation to AMR in horses [17, 35] and represent an increasing problem for public health [33].

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Horse can act as reservoirs of antimicrobial resistance bacteria [10] and genetic factors of antimicrobial resistance which also have significant health implications for their population [20]. Several studies have reported severe infections in horses caused by multidrug resistance (MDR) ESBL-producing Enterobacteriaceae [34]. MDR-ESBL bacterial infections have become a major health concern and cause of treatment failures. These infections could become a critical problem in equine medicine due to the limited number of antimicrobial drugs for use in this species [20]. ESBL-producing *E. coli* (ESBLEC) have been reported in uterine, ocular, soft-tissue, colic, and wound infections in horses [20, 34]. Some studies have reported AMR in horses in Japan [19, 25, 32, 37]. However, there are no previous reports of ESBLEC isolated from horses in Japan. For these reasons, we conducted a study to investigate the occurrence of ESBLEC isolated from feces in healthy racehorses in Japan.

## Materials and Methods

### *Isolation of ESBL-producing E. coli*

Feces samples were collected from 147 healthy Thoroughbred racehorses by equine veterinarians at the Japan Racing Association (JRA) (103 samples from Miho Training Center and 44 samples from Ritto Training Center) between March 2017 and April 2018. Feces samples of freshly passed materials were collected from each horse and stored in sterile plastic bags. Samples were transported directly to our laboratory in a cooling box. ESBLEC were screened based on recommendations from the Clinical Laboratory Standard Institute (CLSI) guidelines [7] with minor modifications. Samples were subcultured in trypticase soy broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and incubated for 24 hr at 37°C. Each sample was cultivated onto MacConkey agar (Nissui Pharmaceutical Co., Ltd.) supplemented with 1 µg/ml cefotaxime (CTX) (Duchefa Biochemie B.V. Haarlem, North Holland, the Netherlands) and was incubated for 24 hr at 37°C for colony isolation. In general, for each sample at least three colonies with typical *E. coli* morphology were picked from MacConkey agar, purified onto trypticase soy agar (Nissui Pharmaceutical Co., Ltd.) supplemented with 1 µg/ml CTX, and incubated for 24 hr at 37°C. Selected isolates were confirmed to the species level using MALDI-TOF MS (Bruker, Billerica, MA, U.S.A.). All the presumptive ESBLECs were further analyzed by polymerase chain reaction (PCR) and confirmed by DNA sequencing.

### *Detection of ESBL gene and DNA sequencing*

DNA from presumptive ESBLEC isolates was extracted using the alkali boiling method [38]. Specific PCR was

performed to determine the TEM and SHV β-lactamase genes according to Schmiedel *et al.* [28] and CTX-M β-lactamase genes according to Batchelor *et al.* [4]. Multiplex PCR was performed to differentiate CTX-M β-lactamase into five phylogenetic groups according to Woodford *et al.* [36]. All PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) with the same primers as the PCR. DNA sequences were analyzed using BioEdit Sequence Alignment Editor v7.2.6 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and compared with sequences in the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST>). *Klebsiella pneumoniae* ATCC 700603 (*bla*<sub>SHV</sub> positive) and *E. coli* ATCC 25922 were used as positive and negative control type strains, respectively.

### *Phylogenetic group of ESBL-producing E. coli*

Phylogenetic analysis was performed to place ESBLECs into four main phylogenetic groups (A, B1, B2, and D) by PCR targeting genes *chuA* and *yjaA* and the DNA fragment TSPE4.C2 [6].

### *Antimicrobial susceptibility test*

All isolates were tested for susceptibility to twelve antimicrobial agents belonging to seven classes of antibiotic, β-lactam (ampicillin [ABPC], cefazolin [CEZ], cefotaxime [CTX]), aminoglycoside (gentamicin [GM], streptomycin [SM]), tetracycline (tetracycline [TC], oxytetracycline [OTC]), amphenicol (chloramphenicol [CP]), polypeptide (colistin [CL]), quinolone (nalidixic acid [NA], enrofloxacin [ERFX]) and folate antagonist (trimethoprim [TM]), by determining the minimum inhibitory concentration (MIC) of these antibiotics based on recommendations from CLSI guidelines [7]. MICs of antimicrobial agents were determined using the broth microdilution method, and results were interpreted using CLSI criteria. In the case of SM, for which there are no CLSI breakpoints, the results were interpreted based on a report from the Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM) system [24]. Isolates that showed resistance to at least three classes of antimicrobial were considered to have multidrug resistance (MDR) [22]. *E. coli* ATCC 25922 was used as quality control type strain.

### *Conjugation assay*

Thirteen selected isolates were considered as donor strains based on phylogenetic analysis for the conjugation assay. Conjugation was performed based on Phornphisutthimas *et al.* [26] with minor modifications. *E. coli* JM109 (Takara Bio Inc., Kusatsu, Japan), which had resistance to NA (Nal<sup>r</sup>) and formed colorless colonies on MacConkey agar, was used as the recipient strain. All donor (samples)

strains were phenotypically sensitive to NA and formed red colonies on MacConkey agar. Briefly, the donor and recipient strains were grown overnight at 37°C in Luria-Bertani broth (Becton, Dickinson and Co., Le Pont-de-Claix, France). Overnight cultures of donors and recipient strains were mixed in 1:1 ratio and incubated at 37°C for 1 hr without shaking. Serial dilutions of mixed cultures were then plated on onto Luria-Bertani agar (Becton, Dickinson and Co.) supplemented with 50 µg/ml NA and 2 µg/ml CTX and subsequently incubated for 24 hr at 37°C. For all conjugation experiments, the donor strain alone and recipient strain alone were used as controls to ensure the effectiveness of the selective plates used. All of mixed cultures from each conjugation experiment without dilution were also plated onto MacConkey agar supplemented with 50 µg/ml NA and 2 µg/ml CTX. The colorless transconjugant colonies were selected from MacConkey agar plates and cultivated onto MacConkey agar again for purification of transconjugant strains. All transconjugants were confirmed by PCR for genes encoding ESBL production. Each conjugation was performed in triplicate from three independent experiments. Efficiency of conjugation was measured by dividing the number of transconjugants by the number of donors. All transconjugants were tested for antimicrobial susceptibility to ABPC, CEZ, CTX, TC, OTC, SM, and TM by MIC as previously described.

### Statistical analysis

Descriptive statistics were used to analyze the susceptibility profiles to each antimicrobial and the efficiency of conjugation was examined by Excel 2017 (version 15.40; Microsoft, Redmond, WA, U.S.A.).

## Results

In this study, ESBLECs were detected in 12 of 147 (8.2%) healthy Thoroughbred racehorse feces samples. A total of 24 ESBLEC isolates were obtained from samples: 14 isolates from 7 samples at Miho Training Center and 10 isolates from 5 samples at Ritto Training Center. All ESBLECs harbored *bla*<sub>CTX-M-2</sub> (24/24, 100%), and both *bla*<sub>TEM</sub> and *bla*<sub>CTX-M-2</sub> were detected in nine isolates (9/24, 37.5%). Sequencing of all *bla*<sub>TEM</sub> and *bla*<sub>CTX-M-2</sub> amplicons identified them as TEM-1 (100%; non-ESBL) and CTX-M-2 (100%), respectively. Phylogenetic grouping of ESBLECs showed that 37.5% (9/24) belonged to group B2, 33.3% (8/24) belonged to group B1, 20.8% (5/24) belonged to group A, and 8.3% (2/24) belonged to group D (Table 1).

ESBLEC phenotypes were highly resistance to ABPC, CEZ, and CTX (all 100%, 24/24 samples), followed by TM (66.7%, 16/24), SM (62.5%, 15/24), TC (25.0%, 6/24), and OTC (25.0%, 6/24). All isolates were sensitive to CP, NA, ERFX, GM, and CL. The antimicrobial resistance patterns for ESBLECs are shown in Table 1. MDR-ESBLECs, with

**Table 1.** Characterization of extended-spectrum β-lactamase-producing *Escherichia coli* (ESBLECs) and their antimicrobial resistance patterns

Horse No.	Sampling location	β-lactamase genes	Antimicrobial resistance pattern <sup>a</sup>	Phylogenetic group	Code of isolate <sup>b</sup> (n=24)
24	Miho <sup>d</sup>	<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, SM, OTC, TC	A	a <sup>c</sup>
		<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, SM, OTC, TC, TM	A	b
34	Miho	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, TM	B2	a <sup>c</sup>
		<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, SM, TM	B2	b, c
39	Miho	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, SM, TM	B1	a <sup>c</sup> , b
47	Miho	<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, SM, OTC, TC, TM	B1	a <sup>c</sup> , b
55	Miho	<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX	D	a <sup>c</sup>
		<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, OTC, TC	D	b
68	Miho	<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, TM	A	a <sup>c</sup>
78	Miho	<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX	B2	a <sup>c</sup> , b
107	Ritto	<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, SM	B1	a <sup>c</sup>
116	Ritto	<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, SM, TM	B2	a <sup>c</sup> , b
		<i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, TM	B2	c
130	Ritto	<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, SM, TM	A	a <sup>c</sup> , b
131	Ritto	<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX	B2	a <sup>c</sup>
		<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, OTC, TC	B1	b <sup>c</sup>
144	Ritto	<i>bla</i> <sub>CTX-M-2</sub>	ABPC, CEZ, CTX, SM, TM	B1	a <sup>c</sup> , b

<sup>a</sup>ABPC, ampicillin; CEZ, cefazolin; CTX, cefotaxime; SM, streptomycin; TC, tetracycline; OTC, oxytetracycline; TM, trimethoprim. Antimicrobial susceptibility test was interpreted using CLSI criteria and JVARM report. <sup>b</sup>One or more isolates were confirmed as ESBLEC from one sample. <sup>c</sup>Thirteen selected donor isolates in conjugation assay; donor isolate per sample was considered based on phylogenetic group analysis. <sup>d</sup>Miho, Miho Training Center, Japan Racing Association, Ibaraki; Ritto, Ritto Training Center, Japan Racing Association, Shiga.

resistance to at least three classes of antibiotic, were found in 14 (58.3%) isolates.

The conjugation assay was successful in eight of 13 isolates selected based on phylogenetic group analysis, and horizontal transmission was confirmed in all transconjugant strains by detection of *bla*<sub>CTX-M-2</sub> using PCR. The donor and recipient strains were confirmed to have not grown on both of Luria-Bertani agar and MacConkey agar supplemented with NA and CTX. The phenotypes of the donor strains were also not detected on MacConkey agar in the conjugation experiment. Efficiency of conjugation was in the range of  $1.4 \times 10^{-2}$  (sd:  $2.7 \times 10^{-3}$ ) to  $6.7 \times 10^{-5}$  (sd:  $2.8 \times 10^{-5}$ ) per donor cell. All transconjugants showed high resistance to all  $\beta$ -lactam antibiotics, and one transconjugant showed resistance to OTC and TC. MIC results for transconjugants and efficiency conjugation are described in Table 2.

## Discussion

In this study, ESBLECs were isolated from 8.2% of horse feces samples. Interestingly, this finding leads us to question how ESBLECs can exist in racehorses in Japan. As mentioned above, *E. coli* could act as a receptor and donor of resistance genes, and we expected that our study may show the presence of ESBLECs in racehorses in Japan. In equine medicine, few studies have reported ESBL producers in commensal bacteria such as *E. coli* in healthy horses. In the U.K., Maddox *et al.* [20] reported that ESBLECs were detected in 6.3% (42/650) of horse feces samples in 2012. In 2015, de Lagarde *et al.* [8] reported that 29% of premises housed horses shedding ESBL/AmpC  $\beta$ -lactamase producing *E. coli* in France. Additionally, 84% of hospitalized horse feces samples (76/91) reportedly contained ESBLECs in an equine clinic in the Netherlands. The use of cephalosporin as a substance for medical therapy in horses is expected to be a factor that increases the selection of

ESBL producers in Enterobacteriaceae bacteria [9].

CTX-M-2 ESBL was identified as a dominant type in this study. TEM-1  $\beta$ -lactamase was also detected in 37.5% CTX-M-2-producing *E. coli* isolates. These results differ from other reports from France [8], the Netherlands [2], and Denmark [18], which describe CTX-M-1 as the dominant type of ESBL. Our findings are similar to some reports in other host species in Japan. In the period of 2000–2011, Harada and Asai [15] reported that CTX-M-2-producing *E. coli* had become the dominant type of ESBL in beef and dairy cattle, pigs, and broilers in Japan. Recently, Sato *et al.* [27] reported the CTX-M-2 ESBL was dominant in ceftiofur-treated dairy cattle and suggested that improper ceftiofur use in veterinary practice might increase the risk for selection of CTX-M-2-producing *E. coli* in intestinal flora. Shiraki *et al.* [30] suggested in their study that transmission of CTX-M-2-producing *E. coli* might have occurred between cattle and humans through the food chain. ESBL-producing Enterobacteriaceae have been demonstrated to share resistance characteristics among humans, companion animals, and horses [28]. In humans, CTX-M-2 was reported to be the dominant type after CTX-M-14 and CTX-M-15 in the period of 2000–2010 in Japan [14].

CTX-M-2-producing *E. coli* isolates (n=24) were distributed in phylogenetic groups B2 (37.5%), B1 (33.3%), A (20.8%), and D (8.3%). Previously, Asai *et al.* [3] reported that most CTX-M-2-producing *E. coli* isolated from cattle and poultry in Japan belonged to phylogenetic group A. In the Netherlands, CTX-M-2-producing *E. coli* isolated from horse feces samples in an equine clinic were reported to be dominant in phylogenetic group B1 [2]. The proportions of each phylogenetic group differed between host species [31] and geographical locations [12].

Spread of the *bla*<sub>CTX-M-2</sub> gene might be associated with the environmental conditions around racehorses. In our results, all CTX-M-2-producing *E. coli* isolated from Ritto

**Table 2.** Minimum inhibitory concentration of donor and transconjugant, and efficiency of conjugation

Antimicrobials <sup>c</sup>	<i>Escherichia coli</i> JM109	47a		55a		78a		107a		116a		130a		131a		144a	
		D <sup>a</sup>	T	D	T	D	T	D	T	D	T	D	T	D	T	D	T
ABPC	4	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
CEZ	2	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
CTX	<0.125	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
SM	2	2	2	16	2	2	8	>256	2	32	2	>256	2	8	2	>256	2
OTC	2	256	>256	4	2	2	2	4	2	2	4	4	2	4	2	4	2
TC	2	128	128	4	4	4	2	2	2	2	4	4	4	2	2	4	4
TM	1	1	1	0.5	1	1	0.5	4	1	>256	1	>256	1	2	1	>256	1
Efficiency of conjugation (sd) <sup>b</sup>	-	$2.2 \times 10^{-4}$ ( $1.1 \times 10^{-4}$ )	$3.0 \times 10^{-4}$ ( $2.1 \times 10^{-4}$ )	$1.6 \times 10^{-3}$ ( $5.6 \times 10^{-4}$ )	$1.1 \times 10^{-3}$ ( $6.7 \times 10^{-5}$ )	$6.7 \times 10^{-5}$ ( $2.8 \times 10^{-5}$ )	$1.9 \times 10^{-3}$ ( $2.3 \times 10^{-3}$ )	$1.5 \times 10^{-4}$ ( $6.6 \times 10^{-5}$ )	$1.4 \times 10^{-2}$ ( $2.7 \times 10^{-3}$ )								

<sup>a</sup>D: donor, T: transconjugant. <sup>b</sup>The efficiency of conjugation is the number of transconjugants per donor cell, from triplicate analysis in three independent experiments; sd: standard deviation. <sup>c</sup>ABPC, ampicillin; CEZ, cefazolin; CTX, cefotaxime; SM, streptomycin; OTC, oxytetracycline; TC, tetracycline; TM, trimethoprim.

Training Center were identified from horses which came from the same breeding stable (data not shown). We suggest that there is the possibility that transmission of *bla*<sub>CTX-M-2</sub> gene between horses in the same breeding place might occur via fecal material. In addition, Dolejska *et al.* [9] reported that the high level of contamination of ESBL-EC in the stable environment was also affected by flies as a vector of ESBL-EC.

ESBL-ECs showed resistance to all  $\beta$ -lactam antibiotics (100%) tested, followed by TM (66.7%), SM (62.5%), OTC (25.0%), and TC (25.0%). Even though our study does not represent *E. coli* in general, our results may be compared with AMR in *E. coli* from horses in other studies. High resistance to trimethoprim and tetracycline was reported in *E. coli* isolates from horse feces in the U.S.A. [11], Ireland [5], and the U.K. [1, 21]. Furthermore, streptomycin-resistant *E. coli* were reported at high prevalence in horse feces from the U.S.A. [11] and Portugal [23]. Multidrug resistance was found in 58.3% of ESBL-ECs in our study. MDR-ESBL-ECs might be related to antimicrobials currently used in equine medicine in Japan. Although they are not considered to cause disease, MDR-ESBL-ECs may make disease more severe via secondary infections. Moreover, they have been reported in several extraintestinal infections (such as uterine, ocular, soft tissue, and wound infections) and are difficult to treat because of limited numbers of drugs available [20, 34]. Considering the case of MDR strains of Enterobacteriaceae, selection of ESBL producers can be expected by co-selection when using other groups of antimicrobial substances for therapy [9].

Antimicrobial susceptibility pattern and phylogenetic group analysis results showed that CTX-M-2-positive isolates were not clonal. Mobile genetic elements such as plasmids harboring *bla*<sub>CTX-M-2</sub> genes may be spread among *E. coli* derived from horses. Our study also demonstrated horizontal transmission in eight of 13 isolates, with the frequency of transfer being in the range of  $1.4 \times 10^{-2}$  (sd:  $2.7 \times 10^{-3}$ ) to  $6.7 \times 10^{-5}$  (sd:  $2.8 \times 10^{-5}$ ) per donor cell. All transconjugants had resistance to high concentrations of cefotaxime (MIC: >256  $\mu$ g/ml) and one transconjugant (47a) was identified as having resistance to tetracyclines. A limitation of our study was the detection of conjugative plasmids, which confirmed that all transconjugants carried *bla*<sub>CTX-M-2</sub>, by PCR. Nevertheless, this result suggests that in the intestinal tract, conjugal transfer of gene-encoded ESBL (*bla*<sub>CTX-M-2</sub>) might occur [28] and may possibly transfer to other pathogenic bacteria such as *Salmonella* spp. and diarrheagenic *E. coli* [30]. Resistance of *Salmonella enterica* isolated from a horse to ceftiofur (a third-generation cephalosporin) has also been reported at a higher level in the U.S.A. [13].

In conclusion, this study showed that racehorses in

Japan are potential reservoirs of ESBL-ECs. Horse feces may act as a source of ESBL genes for *E. coli* that may be transmitted within the environment of training centers (e.g., other horses, soil, water). Our study also indicated that people working in close contact with racehorses, such as veterinarians, caretakers, and owners, may be exposed to a potential risk of infection with ESBL-ECs carried by racehorses. Continuous monitoring of antimicrobial resistance in commensal bacteria such as *E. coli* is necessary to minimize the spread of ESBL producers in racehorses.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

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